



Faculty of Resource Science and Technology

**PRODUCTION OF PIGMENTS BY
*RHODOTORULA MUCILAGINOSA***

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Production of Pigments by *Rhodotorula mucilaginosa*

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This project is submitted in fulfilment of the requirement for the Degree of Bachelor of
Science with Honours
(Resource Biotechnology)

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DECLARATION

I hereby declare that this thesis entitled "Production of Pigments by *Rhodotorula Mucilaginosa*" is my own work and all sources have been quoted and referred to have been acknowledged by means of complete references. It has been submitted and shall not be submitted to other university or institute of higher learning. This work was done under the supervision of Assoc. Prof. Dr. Cirilo Nolasco Hipolito and submitted to partially fulfill the requirement for the degree of Bachelor of Science with Honours in Resource Biotechnology.



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LIST OF ABBREVIATIONS

PDA	Potato dextrose agar
SHH	Sago hampas hydrolysate
HPLC	High-performance liquid chromatography
<i>R. mucilaginosa</i>	<i>Rhodotorula mucilaginosa</i>
<i>R. rubra</i>	<i>Rhodotorula rubra</i>
β -carotene	Beta-carotene
T	Tons
SEM	Scanning electron microscope
BHT	Butylated hydroxyl toluene
Rpm	Rotation per minute
O.D	Optical density
DCW	Dry cell weight
CFU	Colony forming unit
TLC	Thin layer chromatography
DNS	Dinitro salicylic acid
AMG	Amyloglucosidase
DMSO	Dimethyl sulfoxide

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Production of Pigments by *Rhodotorula mucilaginosa*

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ABSTRACT

Rhodotorula yeast belongs to the family of Cryptococcaceae, a subfamily of Rhodotorulodeae. *Rhodotorula* produces major carotenoid pigments like carotene, torularhodin, and torulene. Carotenoids are important natural pigments. The concentration and type of carotenoids production by genus *Rhodotorula* is influenced by the species of microorganism, the constituents of medium and culture conditions. The aim of this study was to investigate the growth, the production of carotenoids produced by *Rhodotorula mucilaginosa* using sago hampas hydrolysate (SHH). Carotenoid assay was conducted to perform the qualitative determination. The highest biomass (9.6 g/l), carotenoid concentration (8.1 mg/l) and specific productivity (845.9 µg/g.h) were scored by *Rhodotorula mucilaginosa*. The production of carotenoids was carried out for 96 hours at 27 °C. The obtained result has proved and demonstrated the capacity of *R. mucilaginosa* to produce carotenoid.

Key words: *Rhodotorula*, Carotene, Torularhodin, Torulene, Sago hampas hydrolysate

ABSTRAK

Rhodotorula yis tergolong dalam keluarga Cryptococcaceae, subfamili daripada Rhodotorulodeae. *Rhodotorula* menghasilkan pigmen karotenoid utama seperti karotena, torularhodin dan torulene. Karotenoid adalah pigmen semula jadi yang penting. Kepekatan dan jenis pengeluaran karotenoid oleh genus *Rhodotorula* dipengaruhi oleh spesies mikroorganisma, kandungan di dalam media dan keadaan kultur. Tujuan kajian ini adalah untuk mengetahui pertumbuhan, pengeluaran karotenoid yang dihasilkan oleh *Rhodotorula mucilaginosa* menggunakan sago hampas hidrolisat (SHH). Pengenalpastian karotenoid dilakukan dengan menggunakan assay karotenoid. Biojisim tertinggi (9.6 g/l), konsentration karotenoid (8.1 mg/l) dan produktiviti spesifik (845.9 µg/g.h) dihasilkan oleh *Rhodotorula mucilaginosa*. Penghasilan karotenoid mengambil masa selama 96 jam dan suhu dikekalkan pada 27 °C. Keputusan yang diperolehi menunjukkan keupayaan *R. mucilaginosa* menghasilkan karotenoid.

Kata kunci: *Rhodotorula*, Karotena, Torularhodin, Torulene, Sago hampas hidrolisat

1.0 INTRODUCTION

Rhodotorula is characterized as starch-like compounds, can undergo fermentation and without the presence of ballistoconidia (Naghavi *et al.*, 2015). The genera *Rhodotorula* refers to a large group of asporogenous carotenoid-producing yeast, part of Basidiomycota phylum (Postgate, 1994; Moliné *et al.*, 2012).

Rhodotorula species produced main carotenoids of beta-carotene, gamma-carotene, torulene and torularhodin (Ungureanu *et al.*, 2012). The proportion of each of the carotenoid relies on the type of strain and the culture conditions (Moliné *et al.*, 2012). It is easily distinguishable by distinctive yellow, pink, orange or red colonies. Colonies of *Rhodotorula* are characterized as smooth, rapid growing, dull or glistening and sometimes soft, roughened and mucoid (Marova *et al.*, 2011).

Carotenoids are valuable molecules in chemical, pharmaceutical, feed and food industries. They act as the precursors of vitamin A, valuable molecules for antioxidant, colouring, possible involve in tumour inhibiting activity and visual attraction of animals (Johnson & Schroeder, 1995).

According to Oliver & Palou (2000) carotenoid has associated to prevent or protect against some severe human disorders for example heart disease, cataracts, cancer, and macular degeneration. Besides, it has also been successfully used to treat photosensitivity disease, for instance, erythropoietic protoporphyria.

Microbial sources offer a promising method of carotenoid production despite the presence of multiple synthetic and natural carotenoids (Ausich, 1997). The paramount interest of using microbial pigments is because of their natural character and safety purpose, show medicinal properties and provide controllable and predictable yield (Joshi *et*

al., 2003). Frengova and Beshkova (2009) stated that carotenoids produced by microbial is very significant due to the seasonal problem and geographic variability in the marketing and production of some pigments of plant origin. Microbial processes utilize low-cost, and natural carbohydrate sources as substrate bring economic advantages. Yeasts are more convenient as they possess the unicellular nature and has higher growth rate when compared with moulds or algae.

High cost of microbial production is the main drawback during practical exploitation of yeast. Therefore, the production cost could be reduced when the yield of the product is increased, and the less expensive substrate is utilized (Marova *et al.*, 2011). Thus, the aim of this study was to assess the growth and production of carotenoids from *Rhodotorula mucilaginosa* using low-cost carbohydrates sources of sago hampas hydrolysate (SHH).

The objectives of the study were

- To evaluate the growth of *Rhodotorula mucilaginosa* using sago hampas hydrolysate (SHH).
- To produce pigments from *Rhodotorula mucilaginosa*.
- To extract and quantify pigments from *Rhodotorula mucilaginosa*.

2.0 LITERATURE REVIEW

2.1 *Rhodotorula mucilaginosa*

Rhodotorula species is carotenoid biosynthetic yeast. It belongs to Basidiomycota phylum and easily distinguishable as orange/red, yellow or pink colonies (Postgate, 1994). *Rhodotorula* was chosen for the experiments for its advantageous features: smooth colonies are formed with boundaries that were clearly defined and occasionally pseudo mycelium is formed (Krulikovsk *et al.*, 2011).

Rhodotorula mucilaginosa is a species of hetero-basidiomycetous and obligate aerobic yeast (Moore & Breedveld, 1989; Naghavi *et al.*, 2014). It has been effectively used for the production of pigments. *Rhodotorula* pigment has small absorption between 400 to 600 nm and maximum absorption at 200 to 400 nm. Beta-carotene, gamma-carotene, torularhodin, and torulene are the main carotenoids produced by *Rhodotorula* species (Ungureanu *et al.*, 2012).

According to Marova *et al.* (2011), there are three active species of the genus *Rhodotorula* which are *Rhodotorula minuta*, *Rhodotorula glutinis*, and *Rhodotorula mucilaginosa* (previously known as *Rhodotorula rubra*). It has been revealed that a type strain of *Rhodotorula rubra* is, in fact, a strain of *Rhodotorula glutinis* and is also similar to the type strain of *Rhodotorula mucilaginosa*. The colonies are normally pink, orange-red to yellow in colour. After incubated for 72 hours at 25 °C on the yeast malt agar, round or budding oval cells and some rudimentary pseudohyphae were present. However, no ascospores were found when observed under microscope (Chanchay *et al.*, 2012).

2.2 Pigments

The word pigment contained a Latin origin. Initially, it was denoted a colour then in the later extended it was used to refer to coloured objects for example makeup. The word pigment was then used to describe the extracts from diverse vegetable and plant, particularly those used for food colouring in the early of middle ages. In the sense of the biological terminology, the word pigment was referred to the coloured matter appear in plants or animals. Pigments are present in the granules found in the cells or cell membranes suspended in a body fluid or deposits in the tissues (Nigam & Pandey, 2009).

Čarnecká (2009) stated that products include pigments and antibiotics are secondary metabolites. Secondary metabolites refer to pathways and product of metabolism from small molecules that are not crucial to the survival of the cells. The induction of secondary metabolism is related to certain developmental stages or environmental conditions. In most yeast, carotenoid pigments start accumulates in late logarithmic phase and proceeds in stationary phase besides it is very adaptable. Variances between strains of same species and diverse cultivation conditions affect the carotenoid production (Frengova & Beshkova, 2009).

2.2.1 Carotenoids

Carotenoids show increasing economic significance as they are used as nutritional supplements, food colorants, in human therapy and cosmetics as antioxidants (Ungureanu *et al.*, 2012). Carotenoids are important for humans and animals; they involve in the scavenging of oxygen radicals, transformation to vitamin A and enhancement of the immune response (Kiokias & Gordon, 2004).

Carotenoids are the class of hydrocarbons (carotene) which only composed of hydrogen and carbon and their xanthophylls which are oxygenated derivatives (Čarnecká, 2009; Frengova & Beshkova, 2009). They comprised of eight isoprenoid units and joined in the arrangement where the isoprenoid units are reversed at the centre of the molecule. The majority of carotenoids contain a linear C₄₀ hydrocarbon backbone. It includes between 3 and 15 conjugated double bonds (Čarnecká, 2009)

According to Čarnecká (2009), carotenoids have characteristics absorption spectra due to the existence of conjugated polyene system and the distinct functional groups in the molecule. Some double bonds determine the spectral properties of carotenoid. Carotenoids usually absorb light between 400 and 500 nm.

Čarnecká (2009) stated that carotenoids are synthesized by many microorganisms and plants in nature. Acetyl-CoA was demonstrated as the key precursor during carotenoid biosynthesis. There are three steps that are involved in the carotenoid biosynthesis pathway. (i) establishment of isopentyl pyrophosphate (IPP), (ii) establishment of phytoene and finally (iii) cyclization and other reactions of lycopene (Figure 1).

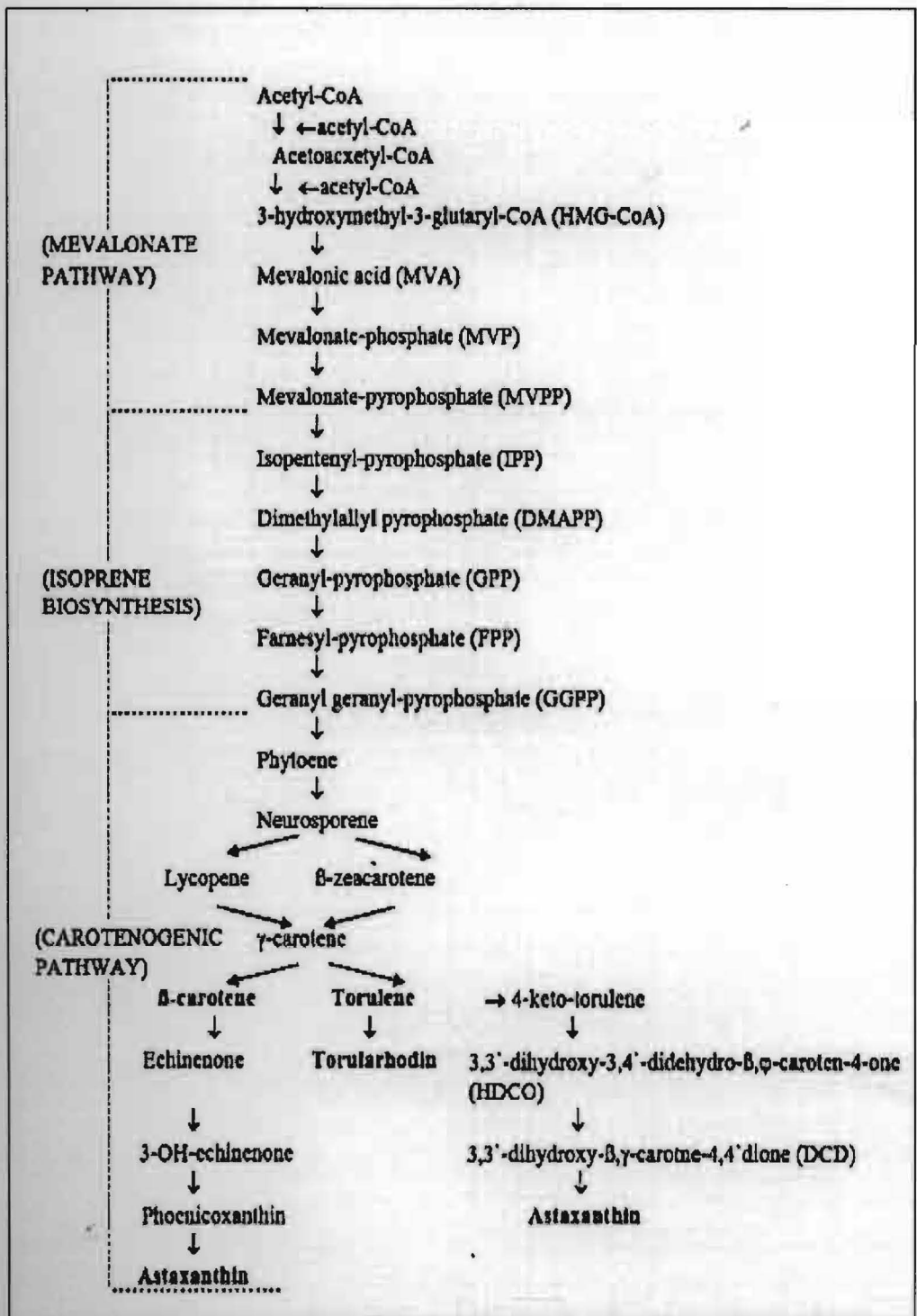


Figure 1: Biosynthetic pathways for *Rhodotorula* and astaxanthin in *P. rhodozyma/X. dendrorhous* (Frengova & Beshkova, 2009).

Rhodotorula species is carotenoid producing yeasts. The main carotenoids were identified as β -carotene, torularhodin, torulene, and γ -carotene (Ungureanu *et al.*, 2012).

Figure 2 shows the chemical structures of β -carotene and torularhodin.

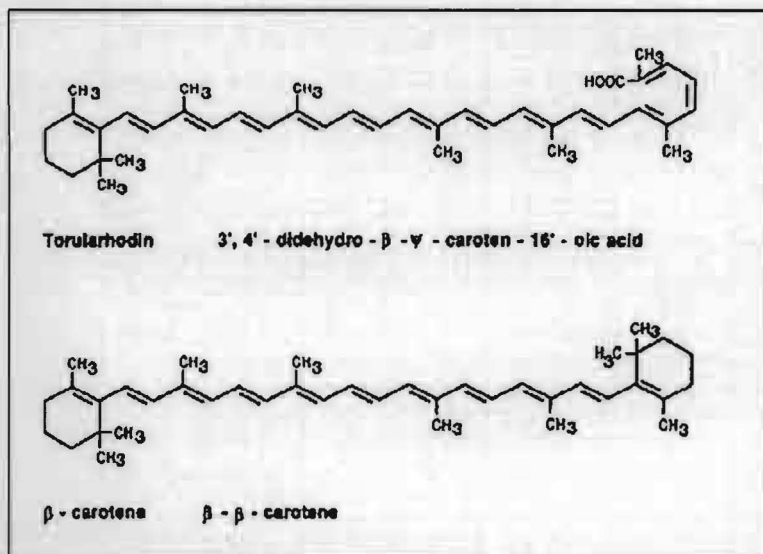


Figure 2: Chemical structure of torularhodin and β -carotene (Moore & Breedveld, 1989).

β -carotene is an orange-yellow pigment. It is an effective quencher of singlet oxygen and is commonly found in animal and plant tissues. β -carotene is used as an additive in food, cosmetic, pharmaceutical and feed products. Moreover, due to its colorant it is used in antioxidant and anticancer activities (Moore & Breedveld, 1989; Malisorn & Suntornsuk, 2009).

Torularhodin (3', 4'-didehydro- β , γ -caroten-16'-oic acid) is present as caroteno-protein in *Rhodotorula mucilaginosa*. Torularhodin is a complex that would be expected to be more stable and water soluble than free carotenoid (Moore & Breedveld, 1989; Ungureanu *et al.*, 2012). Torularhodin is unique carotenoid consists of a carboxyl terminal group as powerful antioxidant included in drug and food formulations (Ungureanu *et al.*, 2012).

Table 1 indicated the amount of carotenoids in wild strain and a mutant strain of *Rhodotorula rubra*. After the growth had ended, the amount of carotenoid reached a maximum. Mutagenically treated *Rhodotorula rubra* GED8 actively synthesized carotenoids when cultivated in synthetic media containing carbon carriers like glucose, sucrose and galactose (Frengova & Beshkova, 2009).

Table 1: Total carotenoids and concentration of individual pigments synthesized by wild strain *R. rubra* GED8 and mutant strain *R. rubra* 56-13 (Frengova & Beshkova, 2009).

Yeast	Total carotenoids		Proportion (β -carotene:torulene: torularhodin, %)
	[mg l ⁻¹ culture fluid]	[μ g g ⁻¹ dry cells]	
<i>Rhodotorula rubra</i> GED8*	2.9 \pm 0.19	194 \pm 5.57	26:30:42
<i>Rhodotorula rubra</i> 56-13*	8.7 \pm 0.25	658 \pm 7.00	63:29:7
<i>Rhodotorula rubra</i> GED8**	5.5 \pm 0.16	305 \pm 8.66	33:28:38
<i>Rhodotorula rubra</i> 56-13**	15.7 \pm 0.31	946 \pm 5.00	71:24:4

* *R. rubra* GED8 and *R. rubra* 56-13 were grown as monocultures in glucose medium.
 ** *R. rubra* GED8 and *R. rubra* 56-13 were grown as mixed cultures with yogurt bacteria (*L. bulgaricus* 2-11 + *S. thermophilus* HA15).
 The concentrations of total carotenoids are the maximum reached on the respective day.

2.3 Sago hampas

In Sarawak, sago starch processing industries generate numerous agro-residues. Sago residue contains solid and also liquid materials (Awg-Adeni *et al.*, 2010). According to Bujang *et al.* (1996) it has been estimated that a single sago starch processing mill produced approximately 7 tons (t) of sago hampas daily. Recently, agro-residues together with the wastewater were washed off into a nearby stream and deposited with factory's compound. The releasing of wastewater could lead to serious environmental problems. Sago wastewater contains high organic material ('hampas'), biological oxygen demand (BOD), and chemical oxygen demand (COD) (Awg-Adeni *et al.*, 2010).

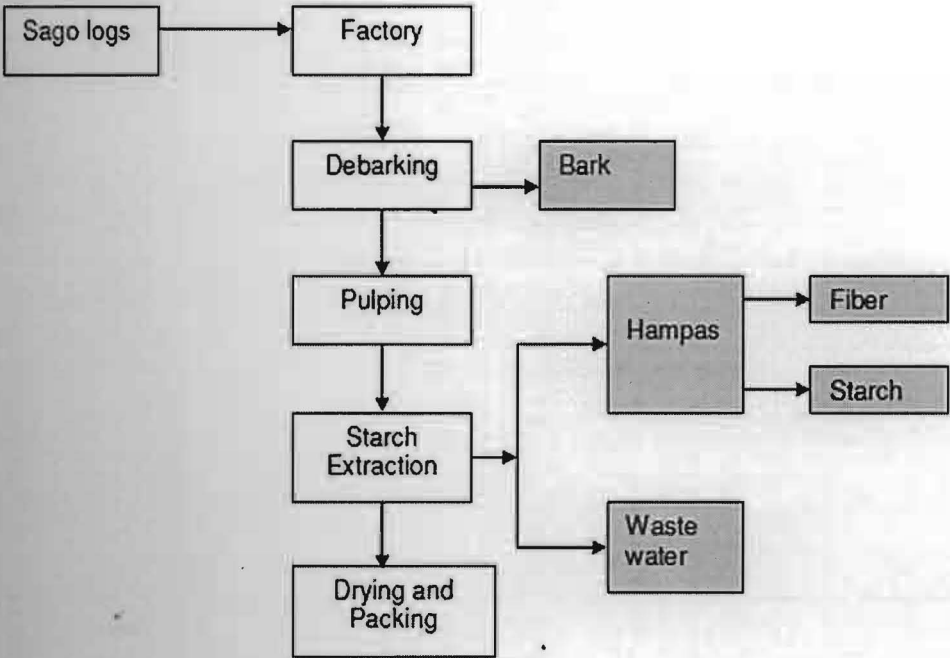


Figure 3: Schematic flow diagram for the processing of sago (Yean & Lan, 1993).

Figure 3 shows the schematic flow diagram for processing of sago. The exploitation of agro-residues offers promising materials resources. For example, sago hampas (fibrous by-products results of sieving and crushing), sago bark (peelings obtain

from initial processing) and sago wastewater can be used for sustainable development and global environmental conservation (Awg-Adeni *et al.*, 2010).

Sago hampas is a lignocellulosic starchy by-product. It is generated from the pith of the *Metroxylon sagu*. In Malaysia, Sarawak is known as the largest sago-growing areas. Currently, it is the biggest exporter of sago starch. Sago hampas on dry basis contains 58% starch, 23% cellulose, 9.2% hemicellulose, and 4% lignin (Linggang *et al.*, 2012). According to Awg-Adeni *et al.* (2010), hampas contains around 66 % of starch, 14 % of fibre on a dry weight basis of which it is made up of approximately 25 % of lignin. Table 2 shows the composition analysis of sago hampas on a dry basis.

Table 2: Composition analysis of sago hampas (Awg-Adeni *et al.* (2010).

Composition	% (dry basis)
Starch	30–45
Moisture	5–7
Ash	3–4
Protein	1
Fiber	30–35
Fat	ND
pH	4.6–4.7

ND: not detected.

The dried powder form of sago hampas has shown its possibility to convert into fermentable sugar via enzymatic hydrolysis and acid treatment. According to Kumoro *et al.* (2008), a total of 0.6234 glucose per hampas (g g^{-1}) was produced after 120 minutes when hydrolyzed with 1.5 M sulphuric acid at 90 °C. After 30 minutes, reaction time, when the hampas was hydrolysed by glucoamylase it yield 0.5646 glucose per hampas (g g^{-1}). It is more effective to enhance the sugar yield when higher temperature and higher acid concentration were used rather than extend the period of hydrolysis reaction time. Higher

temperature offers greater energy to destroy the linkage of fibrous sago hampas (Awg-Adeni *et al.*, 2010).

According to Awg-Adeni *et al.* (2010), sago starch has a high demand in the market due to its possibility to produce food, polymer, textile and pharmaceutical industry. Therefore, bioconversion of hampas into economically useful products, for instance, fermentable sugar, feed, enzymes and compost helps to solve environmental problems like agro-residues were washed off into nearby streams.

The presence of suitable carbon source is crucial for the synthesis of carotenoids. *Rhodotorula* has the potential as a natural source of carotenoids. However, there is a limit use of *Rhodotorula* due to the high cost of production. Agro-industrial raw materials may be used as low-cost carbohydrates sources (Table 3) (Čárnecká, 2009). Agro-industrial waste generate serious environmental problem and hence bioconversion of agro-residues into value-added products help to minimize the environmental issue and cost of production.

Table 3: Comparison of carotenoid production and cell mass of *Rhodotorula* species grown on altered waste substrates as carbon sources (Čárnecká, 2009).

Rhodotorula species	Carbon source	Cultivation process	Cell mass (g/l)	Carotenoids (mg/g CDW)	Carotenoids (mg/l culture)
<i>R. glutinis</i> ATCC 26085	glucose	batch		0.206	
<i>R. glutinis</i> 32	glucose	batch	23.9	5.4	129.0
<i>R. glutinis</i> 32	sugar cane molasses	fed-batch	78.0	2.36	183.0
<i>R. glutinis</i> DBVPG 3853 <i>D. castellii</i> DBVPG 3503	corn syrup	fed-batch	15.3	0.535	8.20
<i>R. glutinis</i> TISTR	hydrolyzed mung bean waste flour	batch	10.35	0.345	3.48
<i>R. glutinis</i> 22P <i>L. helveticus</i> 12A	whey ultrafiltrate	batch	30.2	0.268	8.10
<i>R. mucilaginosa</i> NRRL-2502	sugar-beet molasses	batch	4.2	21.20	89.0
<i>R. mucilaginosa</i> NRRR-2502	whey	batch	2.4	29.2	70.0

3.0 MATERIALS AND METHODS

3.1 Microorganisms

Rhodotorula mucilaginosa strain that was used was a strain locally isolated in Universiti Malaysia Sarawak (UNIMAS) laboratory. Potato dextrose agar was used to maintain the yeast culture. The culture was stored in a refrigerator at 4 °C after incubation at 30 °C for 24 to 72 hours. The purity of the culture was regulatory checked. The yeast strain was activated weekly (Yehia *et al.*, 2013).

3.2 Preparation of Media

3.2.1 Maintaining media

Potato dextrose agar (PDA) was recommended by Griffith *et al.*, (2007) and was used for the cultivation and isolation of yeast. PDA enabled healthy growth, sporulation and pigmentation for a wide variety of fungal taxa. The medium have the following composition (g/l):

-Potato extract (4.0), Dextrose (20.0), Agar (15.0)

All media used throughout this study was dissolved in distilled water. The medium was prepared and autoclaved at 121 °C for 20 minutes.

3.3 Preparation of sago hampas hydrolysate (SHH)

Sago hampas was obtained from Herdsen Sago Mill in Sarawak, Malaysia. Prior to composition analysis, the sago hampas was oven dried at 90 °C for 24 hours before grounded to pass through a 1 mm sieves. Dried sago hampas were analysed for moisture

content. Therefore, a suitable amount of buffer was added to carry out the enzymatic hydrolysis process (Awg-Adeni *et al.*, 2012).

A suspension of sago hampas, 1% (w/v) was dissolved in distilled water. The pH of the suspension was adjusted to pH 6.5. Enzymatic hydrolysis was performed using 0.5 µl Termamyl (a thermostable α- amylase from *Bacillus licheniformis*, 120 KNU/g) (Science Technics, Malaysia) per gram of sago hampas for the purpose of liquefaction. The suspension was stirred and heated for 2 hours at 90-95 °C. Next, 0.6 µl amyloglucosidase (AMG) (Science Technics, Malaysia) per gram of sago hampas was added. The pH was adjusted to 4.5 for the purpose of saccharification, and the suspension was incubated on a shaker at 60-63 °C for 24 hours.

Figure 4 showed the sago hampas suspension obtained after saccharification. The hydrolysate obtained was separated from the residual lignocellulosic fiber using 100 mesh sieve filter by filtration. It was then centrifuged at 8000 rpm for 20 minutes at 4 °C. The supernatant was referred as sago hampas hydrolysate (SHH). It was harvested and analysed for glucose content using high-performance liquid chromatography (HPLC). The pellet (lignocellulosic fiber) was dried in the oven before being observed for a physical structure under Scanning Electron Microscope (SEM) (Awg-Adeni *et al.*, 2012).



Figure 4: Sago hampas suspension.

3.4 Cultivation of yeast for carotenoid production

3.4.1 Inoculum preparation

A loop full of an active (24 hours) *Rhodotorula mucilaginosa* cultivated on the potato dextrose agar was transferred to a universal bottle contained 40 g/l glucose and 5g/l yeast extract. The strain was incubated in an incubator for 24 hours at 36 °C. After that, the activated *R. mucilaginosa* was transferred into 200 mL growth medium containing 8 g of glucose and 1 g of yeast extract. The cell were incubated for 12 hours at room temperature as the inoculum (Figure 5). *Rhodotorula* was prepared in 10^4 CFU/ml. The medium turned into slightly cloudy as the number of yeasts was increased. Centrifugation of cells was performed at 3000 rpm for 10 minutes to obtain a cell pellet. Sterilised distilled water was used to rinse the cell pellet before transferred into the bioreactor.

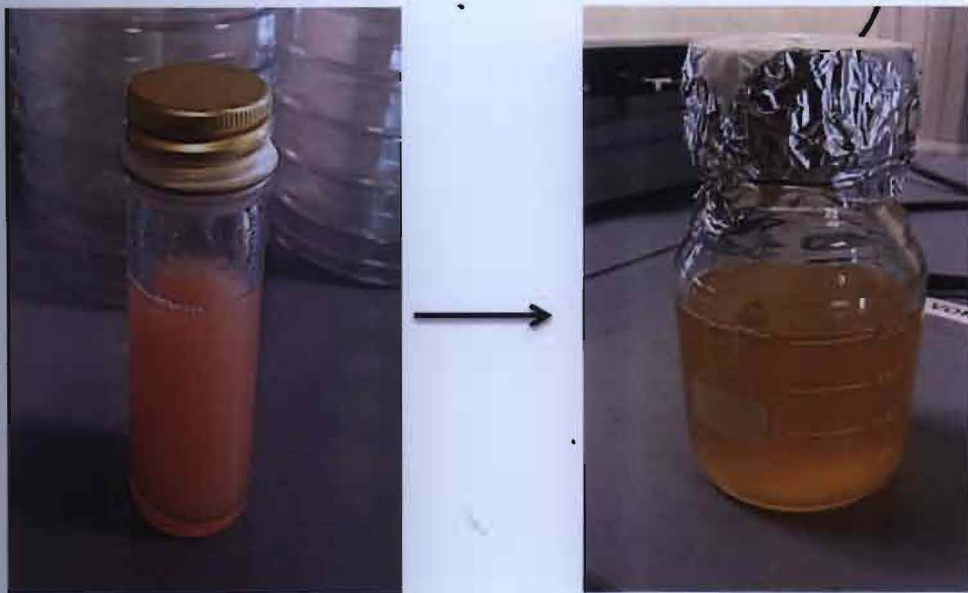


Figure 5: Inoculum preparation